REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget. Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (*Leave Blank*)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	See Title Page	1 February 1987 to 31 January 1991

4. TITLE AND SUBTITLE

~~Use Title on Reprint~~

5. FUNDING NUMBERS

N00014-87-C-0146
0R0A444C-
43051
43051S4

6. AUTHOR(S)

See Individual Articles

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Vanderbilt University
Nashville TN 37332
(615-322-2786)

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Office of Naval Research
800 North Quincy Street
Arlington, VA 22217-5660

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Each Paper Summarized on first page. Journal articles submitted as contract reports. All work performed under Government contract.

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (*Maximum 200 words*)

See first page of Article

DTIC
ELECTE
JAN 1 6 1996
F

19960103 227

14. SUBJECT TERMS

Free Electron Lasers Medicine Biology
Biomedical Instrumentation
Energy Cells

15. NUMBER OF PAGES

00

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std 239-18
298-102

Accepted for Publication in
Lasers in Surgery and Medicine

IV-C.

Role of Immersion Refractometry for Investigating Laser Induced Effects in Cells

Jerri Tribble, M.S., John Kozub, B.S., Al Ali, M.D.,
Robert Ossoff, M.D., and Glenn Edwards, Ph.D.

Department of Otolaryngology (JT,AA,RO) and
Department of Physics and Astronomy (JT,JK,GE)
Vanderbilt University
Nashville, Tennessee

ACKNOWLEDGMENTS

E. coli Cla was a gift from J.M. Davidson, Vanderbilt University. This research has been supported by the University Research Council and Natural Science Committee at Vanderbilt University and by the Office of Naval Research through Contract Number ONR NOOO14-87-C-0146.

Accesion For
NTIS CRA&I ☑
DTIC TAB ☐
Unannounced ☐
Justification
By
Distribution/
Availability Codes
Dist | Avail and/or Special
A-1

KEYWORDS

Bacteriostasis, phototherapy, porphyrin complexes, Soret Band, tunable-dye laser

ABSTRACT

The broad background of scattered light observed in spectra of cell suspensions is reduced by factors of up to twenty by immersion refractometry allowing for improved spectroscopic determination of the absorption properties of cells in the 325 to 820 nm range. Refractive-index matched spectra of E.coli C1a exhibit a set of resonant features near 422, 561, and 582 nm. Exposure wavelengths are chosen based on this spectrum and cell viability is investigated in E.coli suspensions exposed to 350, 400, 422, 440, and 700 nm radiation delivered in nanosecond pulses with total doses from 500 millijoules to 60 Joules. We observe a loss in cell viability for doses greater than 1 Joule at 422 nm and for all doses at other wavelengths; exposures of less than 1 Joule at 422 nm enhance growth. Excluding exposures at wavelengths within the resonant feature, longer wavelengths are less effective at reducing the viability of E.coli C1a. This indicates the occurrence of at least two absorption processes.

INTRODUCTION

Recent advances in laser technology have resulted in broadly tunable radiation sources in the ultraviolet, visible, and infrared wavelength ranges with pulsed or continuous wave operation (1,2). These sources provide a wide range of wavelengths and pulse lengths for investigating laser induced effects in biological systems. The choice of wavelength is often strongly influenced by the absorption characteristics of cells, tissues, or their components, which in principle can be measured

with standard spectroscopic techniques (3). In many cases, however, the spectroscopic measurements are dominated by scattered light, obscuring the absorption spectra.

In general terms, the mechanisms for the absorption of light can be described as photochemical or photothermal in nature and, in some cases, result in significant biological effects (4,5). In the past, most biomedical applications of lasers have been attributed to photothermal mechanisms (6,7). The biomedical applications of broadly tunable and pulsed lasers are currently under investigation with the aim of developing more selective effects based on photothermal or possibly photochemical mechanisms. A systematic investigation of the effects of laser light on a given biological system will be based on knowledge of the absorption characteristics throughout the accessible wavelength range. It must be recognized, however, that while spectroscopic analysis identifies mechanisms for depositing energy into a biological system, it is not necessarily the case that these mechanisms will result in biomedical effects.

Relative to x rays, which generally induce major damage in molecular structure, UV wavelengths can be quite selective in their interactions with biological molecules. It has become common practice to view the 300 nm wavelength as the demarcation between far-UV (<300 nm) and near-UV (300-380 nm) radiation (4). The far-UV is characterized by a strong absorption by nucleic acids centered near 260 nm and a weaker, but still significant, absorption by proteins centered near 280 nm. Investigations of

the viability of E.coli irradiated with near-UV to visible (<550 nm) radiation suggest additional absorption bands in this wavelength range (8).

In this study we develop a protocol for investigating the effect of laser light on the viability of cells. To reduce light scattering in absorption measurements we implement the technique of immersion refractometry which is based on matching the refractive index of the suspending solution to that of the cell membrane (9). Guided by the absorption spectra we select a set of wavelengths for laser irradiation. Cells suspended in Luria broth are exposed to increasing doses of nanosecond pulses of near ultraviolet and visible laser light. The subsequent viability of the cells is monitored by two techniques, plate counting and absorption measurements of cell cultures. We have chosen E.coli as the initial system for investigation.

MATERIALS AND METHODS

Spectroscopy

Measurements of the optical properties of cell suspensions are typically obscured by a dominant background of scattered light. Biological applications of immersion refractometry reduce light scattering by suspending the cells in a protein solution that matches the refractive index of the cell membrane (9). By varying the protein content of the suspending solution, one can minimize the scatter and thus optimize the determination of the absorption spectra. Bovine serum albumin (BSA) was purchased

from Sigma Chemical Company and initially prepared as a stock solution of 45% weight/volume in distilled water. The solution was manually stirred and then clarified by centrifuging at 1000g for 30 minutes and had a refractive index of greater than 1.42. Refractive indices were measured with a Bausch & Lomb Abbe-3L refractometer.

E.coli C1a to be used in the immersion refractometry measurements were grown overnight in Luria broth and centrifuged for five minutes at 10,000g. The pellets of wet packed cells (wpc) were resuspended to a concentration of 100 mg-wpc/ml. The cell suspension was diluted 1:9 with BSA solution yielding a concentration of 10^8 cells/ml. Absorbance was measured with a Milton Roy Spectronic 601 spectrophotometer.

Spectra of optimally index-matched cells were taken with a Hewlett Packard 8452 multidiode array spectrometer with 2 nm resolution. Some of the spectra reveal artifactual lines due to the deuterium source (10); these lines lie near, with decreasing intensity, 656, 486, and 580 nm. The residual background of scattered light was modeled with the function:

$$A = \frac{C}{\lambda^p}$$

where A is the absorbance, λ is the wavelength of light, and C and p are parameters to be fit with a linear least squares algorithm. The scattering background was subtracted and the remaining resonant features were modeled with Gaussian lineshapes.

Laser Irradiation

Laser light was produced by a Quantel 571C Nd:YAG pumped TDL50 dye laser system with doubling crystals. Pulses of 6-8 ns duration at 10 Hz repetition rate had an energy per pulse of approximately 3 mJ/pulse as determined with an Ophir 30 AP calorimeter. Bacteria were kept on ice prior to and after exposure, but during exposure were at room temperature in 20 microliter aliquots in a microcentrifuge tube. The laser beam is approximately 5 mm in diameter and the sample was centered in the beam.

Bacteriology

E.coli C1a was grown to the stationary phase in Luria broth at 37 C and put on ice for laser irradiation. After irradiation the viability of the exposed and control cells was assayed by two techniques. In one technique growth was monitored by measuring the absorbance at 600 nm at 20 minute intervals. The log phase of the growth curve was fit with an exponential function in the standard way (11), the ratio of the concentration of the exposed cells relative to the control cells was extrapolated to the time of exposure to yield a relative initial concentration. The absorbance method is susceptible to a laser induced growth delay being misinterpreted as laser induced bacteriostasis, i.e. a significant growth delay will be interpreted as an erroneously large reduction in the number of viable cells.

The other technique determines cell viability by counting colonies on agar plates. Although this technique is less precise

than absorption measurements, it has the attribute of being less sensitive to variations in growth delay.

RESULTS

Absorption spectra for E.coli in the 325 to 820 nm range are presented in Figure 1. Figure 1a refers to E.coli suspended in saline and exhibits a broad shoulder near 350 nm and domination of the spectrum by scattered light. Figure 1b presents the absorption spectrum for E.coli suspended in the BSA solution that optimally matches the refractive index, as shown in figure 2. Note that in comparing figures 1a and 1b, the index matching reduces the light scattering background by a factor of 20 at 350 nm.

The results of the curve fitting are presented in figure 3. The residual light scattering as shown in figure 1b is modeled in figure 3a and a resonant feature centered near 422 nm is evident. The p value for fitting the spectrum shown in figure 1a is 2.11, in agreement with earlier work (12). For the index matched solution, the p value reduces to 1.67.

In light of these spectroscopic results, we selected 350, 400, 422, 440, and 700 nm as wavelengths for further investigation. tRNA (13), flavins (14,15), cytochrome (16), and NADH (17) contribute to the broad absorption near 350 nm. In addition, absorption measurements of water (18) indicate a broad, intense band centered at terahertz frequencies that also contributes to the absorption at 350 nm. Absorption measurements of Luria broth

(data not shown) reveal no resonant features in this frequency range. The intensity pattern of the 422, 582 and 561 nm features are reminiscent of the Soret, α, and β bands, respectively, observed in porphyrin complexes (16,19); 422 nm was chosen since it corresponds to the dominant resonant feature. 400 and 440 nm were chosen to demarcate the resonant feature. 700 nm was chosen as a nonresonant wavelength in the red. Figure 4 presents the percentage kill for E.coli exposed to increasing doses of radiation at these wavelengths. Figure 5 presents the percentage kill at a dose of approximately 4 J as a function of wavelength. With the exception of exposures to 350 nm, there were no significant differences between plate counting and absorption measurements for determining cell viability. Exposures to 350 nm indicate a radiation induced growth delay (5). Cells exposed to 422 nm radiation did repeatably indicate a growth enhancement for doses of less than 1 joule. There have been previous reports of stimulatory effects (20).

DISCUSSION

As shown in figures 1 and 2, immersion refractometry successfully reduces light scattering and significantly improves spectroscopic measurements of the absorption properties of E.coli suspensions. We have recently extended these studies to other cell lines with similar success.

With regards to biological effects of laser irradiation of E.coli, the cell viability studies summarized in figures 4 and 5 indicate the occurrence of at least two absorption processes.

More specifically, one process is indicated by the growth enhancement due to low doses of 422 nm radiation. A second process is indicated at larger doses where increasing dose corresponds to increasing lethality and, excluding the 422 nm data, cell viability is less effectively reduced with longer wavelength radiation. This second process is in line with conclusions drawn from previously observed action spectra of E. coli where nucleic acids have been assigned as the principle chromophore (4,5,8). At lower doses and for wavelengths near 422 nm, however, the first process is apparent and the net effect on cell viability is opposite to that of the second mechanism. Furthermore, in our studies with the nanosecond pulsed laser, lethality is achieved at significantly lower doses than previously reported (4,5). While this is a promising observation for future biomedical investigations, the results discussed above indicate that competing processes occur for irradiation near 422 nm at relatively low doses. This consideration suggests that caution be used with regards to biomedical investigations using wavelengths near the Soret band of porphyrin complexes.

In conclusion, we have reported a systematic investigation of the wavelength dependence of near ultraviolet and visible radiation, delivered in nanosecond pulses, on the growth of E.coli suspensions. Exposure wavelengths were chosen based on improved spectroscopic measurements of the absorption characteristics of cell suspensions where the technique of immersion refractometry has resulted in a significant reduction in the background of scattered light revealing resonant features at 422,

582 and 561 nm. We observe a definite wavelength dependence for the effect of pulsed laser light on the viability of cells that can be reasonably accounted for by spectral assignments and suggests at least two absorption processes. This experimental approach should be of general utility for future investigations of the effects of laser light on cellular systems.

REFERENCES

1. Young M, "Optics and Lasers." (Springer-Verlag, Berlin, 1984).

2. Brau CA, Free-Electron Lasers, Science 1988; 239:1115-1121.

3. Cantor CR, Schimmel PR. "Biophysical Chemistry Part II." (W.H. Freeman and Company, New York, 1980).

4. Jagger J in Wang, SY (ed): "Photochemistry and Photobiology of Nucleic Acids, Volume II: Biology." (Academic Press, New York, 1976) Chapter 4.

5. Jagger J. Near-UV radiation effects on microorganisms. Photochem Photobiol 1981; 34:761-768.

6. Anderson RR, Parrish JA. Selective photothermolysis: precise microsurgery by selective absorption of pulsed radiation. Science 1983; 220:524-527.

7. Hillenkamp F, Pratesi R, Sacchi CA (eds): "Lasers in Biology and Medicine." (Plenum, New York, 1980).

8. Webb RB and Brown SB, Sensitivity of Strains of Escherichia Coli Differing in Repair Capability to Far UV, Near UV and Visible Radiations. Photochem Photobiol 1976; 24:425-432. See Figure 7.

9. Bateman JB, Wagman J, Carstensen EL, Refraction and absorption of light in bacterial suspensions. Kolloid-Zeit 1966; 208:44-58.

10. Moore JH, Davis CC, Coplan MA: "Building Scientific Apparatus." (Addison-Wesley, Don Mills, Ontaria 1983) See figure 4.78.

11. Willett HP. Physiology of Bacterial Growth. In Joklik WK, Willett HP, Amos DB (eds): "Zinsser Microbiology." 17th ed. (Appleton-Century-Crofts, New York, 1980) pages 86-90.

12. Koch AL. Some Calculations on the turbidity of mitochondrial and bacterial suspensions. Biochem Biophys Acta 1961; 51:429-441.

13. Favre A, Michelson AM, Yaniv M. Photochemistry of 4-thiouridine in Escherichia coli transfer RNA(val). J Mol Biol 1971; 58:367-379.

14. Iwatsuki N, Joe CO, Werbin H. Evidence that deoxyribonucleic acid photolyase from bakers yeast is a flavoprotein. Biochemistry 1980; 19(6):1172-1176.

15. Metzler DE. "Biochemistry: the Reactions of Living Cells." (Academic Press, 1977, New York) pages 594, 478.

16. ibid, p.568.

17. ibid, p.478.

18. Jackson JD: "Classical Electrodynamics." 2nd ed. (John-Wiley and Sons, New York, 1975). p. 291.

19. Lemberg R, Barrett J: "Cytochromes." (Academic Press, New York, 1973).

20. Tiphlova O, Karu T. Stimulation of Escherichia coli division by low intensity monochromatic visible light. Photochem Photobiol 1988; 48(4):467-471.

FIGURE LEGENDS

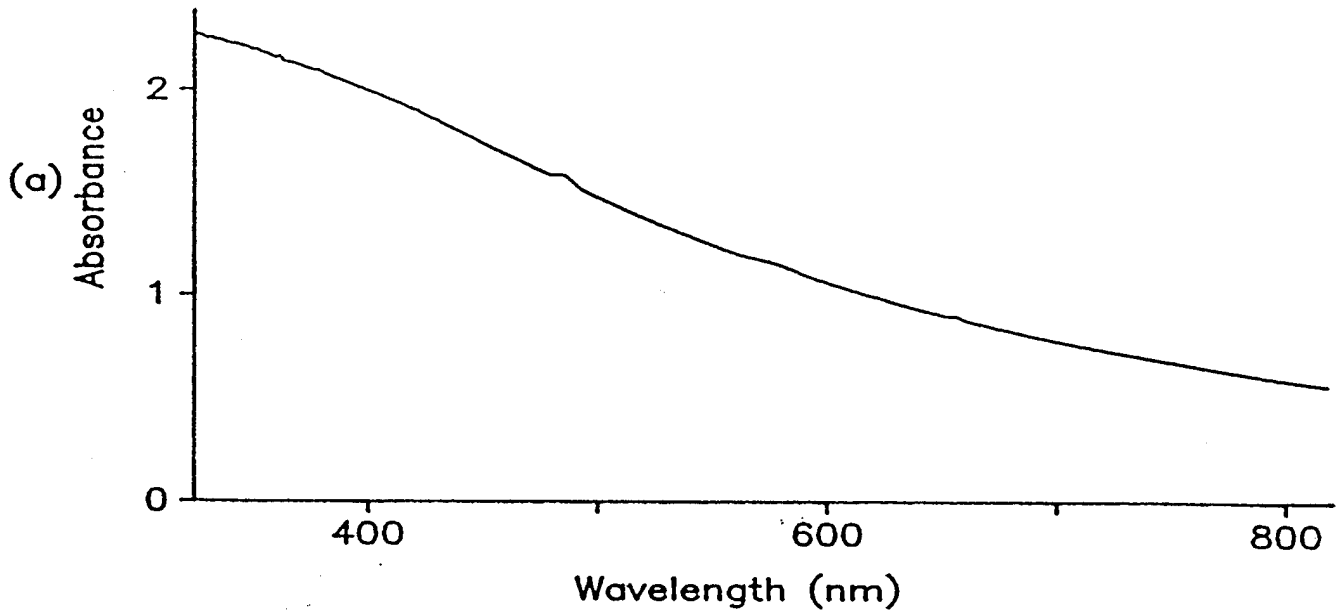
Figure 1. Absorption spectra of E.coli C1a: a) suspended in saline solution exhibiting a shoulder near 350 nm and a broad scattering background, b) suspended in the BSA solution that optimally matches the refractive index of the cell membranes. Resonant features near 422, 582, and 561 nm are revealed due to the reduction in scattered light. Instrumental features occur near 488, 580, and 656 nm (see text); however, the observed intensity of the spectral feature at 582 nm cannot be completely accounted for as an instrumental line.

Figure 2. Optimization of refractive index of suspending solution. Absorption of cell suspensions measured at 589 nm as a function of refractive index of suspending solution. Refractive index varies with BSA concentration. Minimum absorption corresponds to optimally matching the refractive index of the suspending solution to that of the cell membrane.

Figure 3. Curve fitting of absorption spectra. Spectrum shown in figure 1b is fit with a scattering background and resonant feature: a) determination of power law of scattering background, b) Gaussian fit of resonant feature centered at 422 nm.

Figure 4. Wavelength and dose dependence on percentage kill of E.coli: a) exposures to 350, 400, 422, and 700 nm radiation indicating relative effectiveness at reducing cell viability as determined by absorbance measurements of the growth kinetics (see text), b) exposures at 400, 422, and 440 nm indicating growth enhancement for 500 millijoule dose of 422 nm radiation and reduction in cell viability for larger doses. The error in determining percentage kill is approximately 11%.

Figure 5. Percentage kill at a dose of approximately 4 J as a function of irradiation wavelength. The error in determining percentage kill is approximately 11%.


(a)
Absorbance
2
1
0
400
600
800
Wavelength (nm)

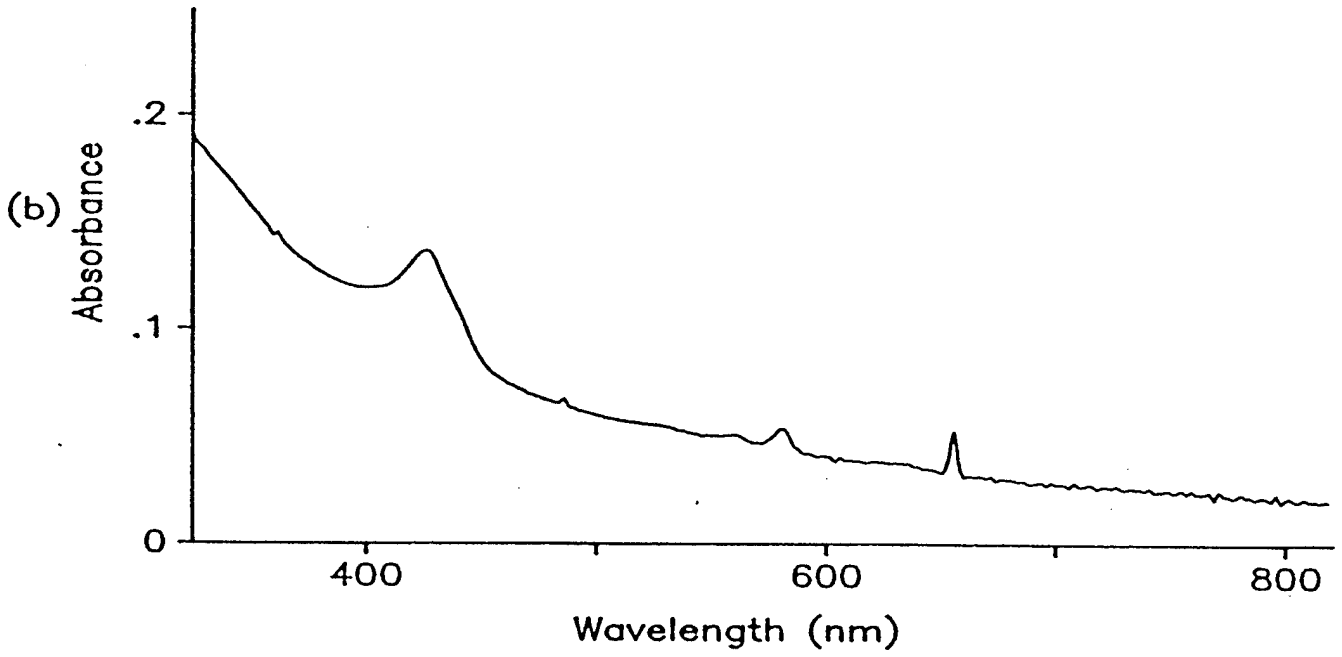

(b)
Absorbance
.2
.1
0
400
600
800
Wavelength (nm)

Figure 1

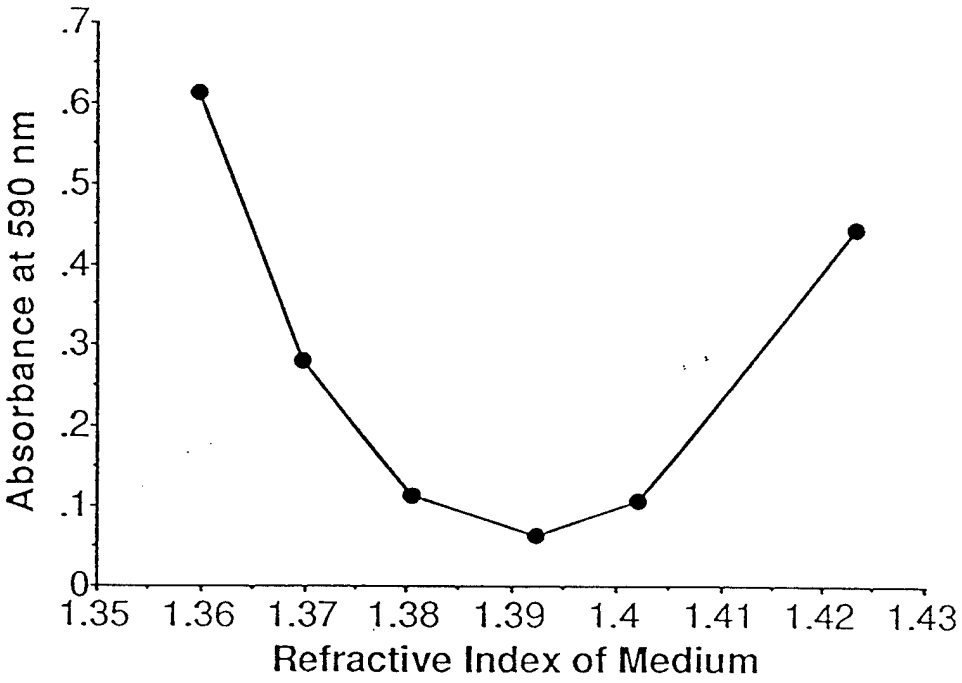
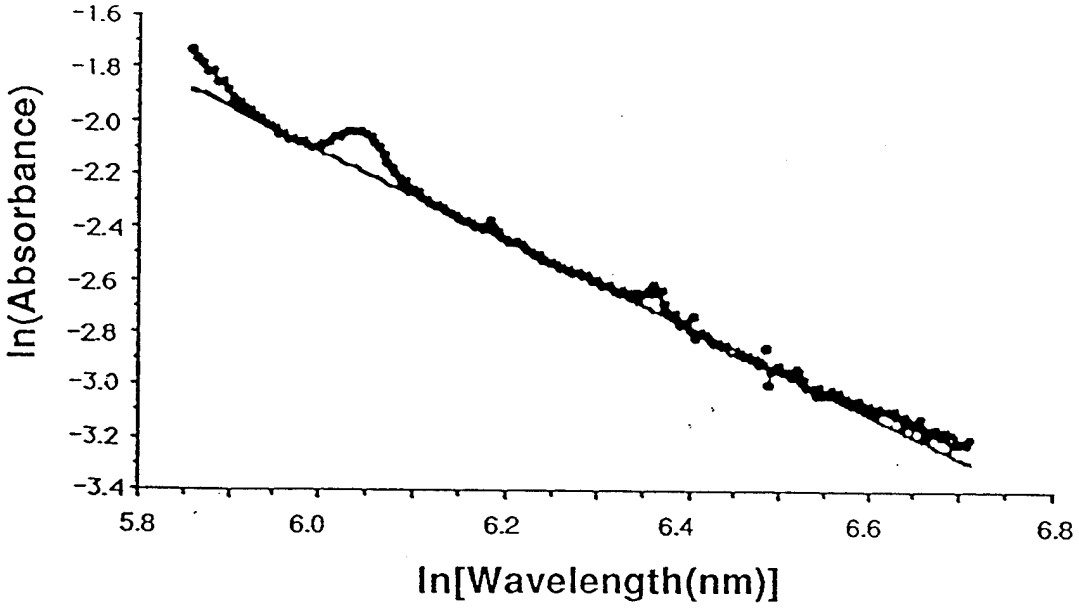

Absorbance at 590 nm
.7
.6
.5
.4
.3
.2
.1
0
1.35
1.36
1.37
1.38
1.39
1.4
1.41
1.42
1.43
Refractive Index of Medium

Figure 2

(a)


-1.6
-1.8
-2.0
-2.2
-2.4
-2.6
-2.8
-3.0
-3.2
-3.4
ln(Absorbance)
5.8
6.0
6.2
6.4
6.6
6.8
ln[Wavelength(nm)]

(b)

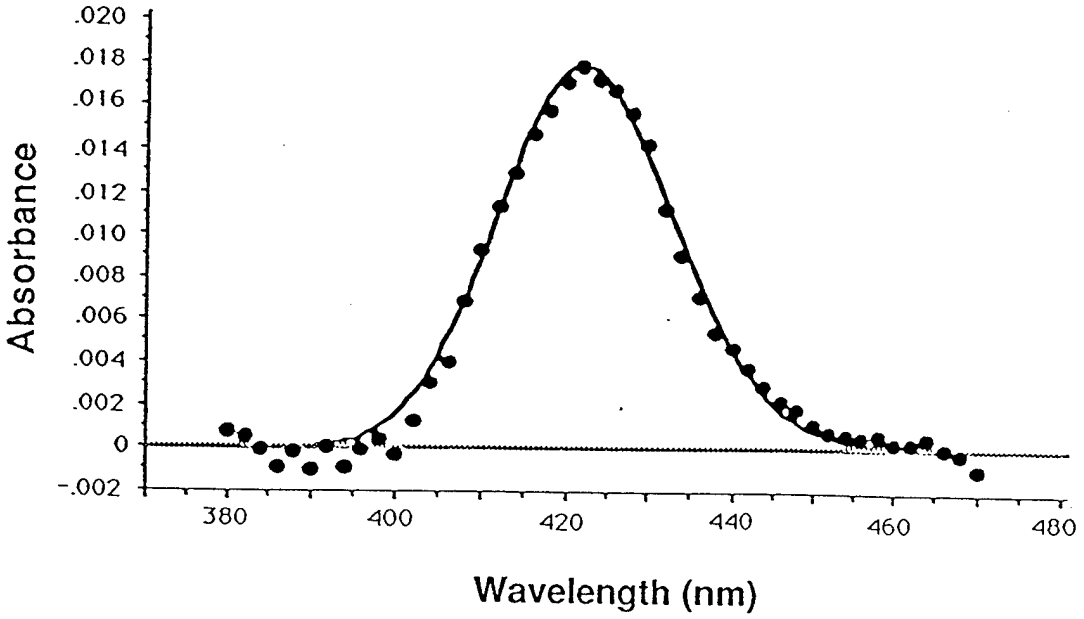
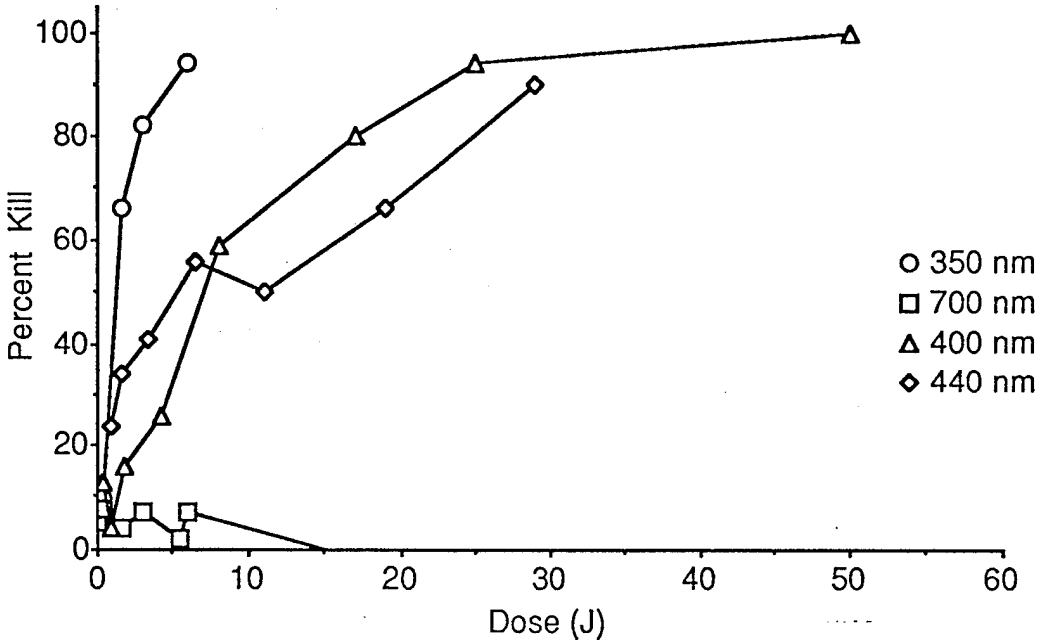

.020
.018
.016
.014
.012
.010
.008
.006
.004
.002
0
-.002
Absorbance
380
400
420
440
460
480
Wavelength (nm)

Figure 3

(a)


100
80
60
40
20
0
Percent Kill
0
10
20
30
40
50
60
Dose (J)
350 nm
700 nm
400 nm
440 nm

(b)

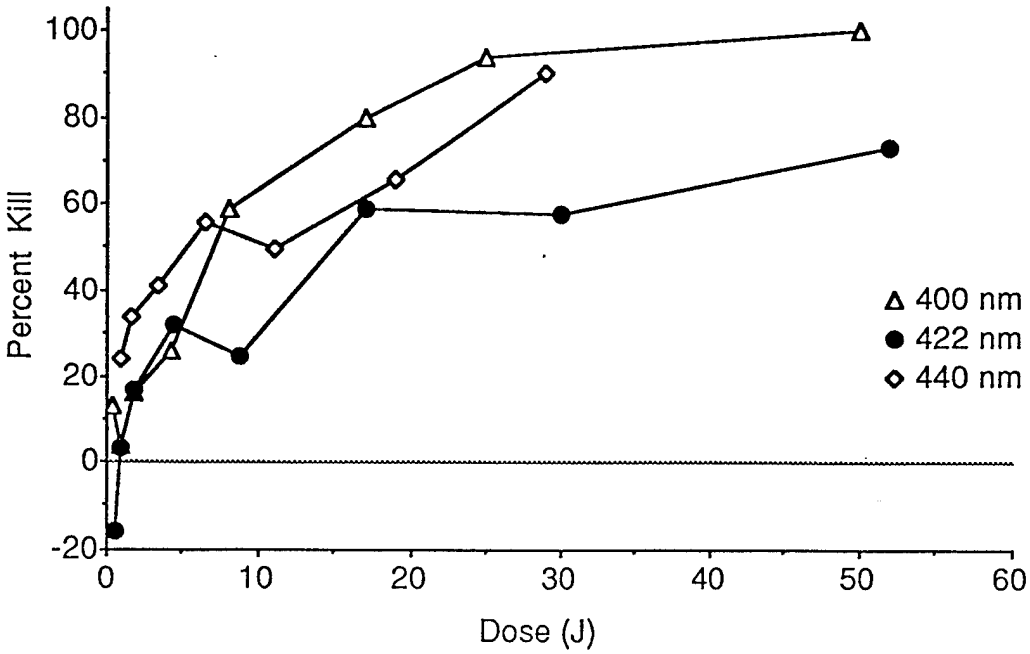

100
80
60
40
20
0
-20
Percent Kill
0
10
20
30
40
50
60
Dose (J)
400 nm
422 nm
440 nm

Figure 4

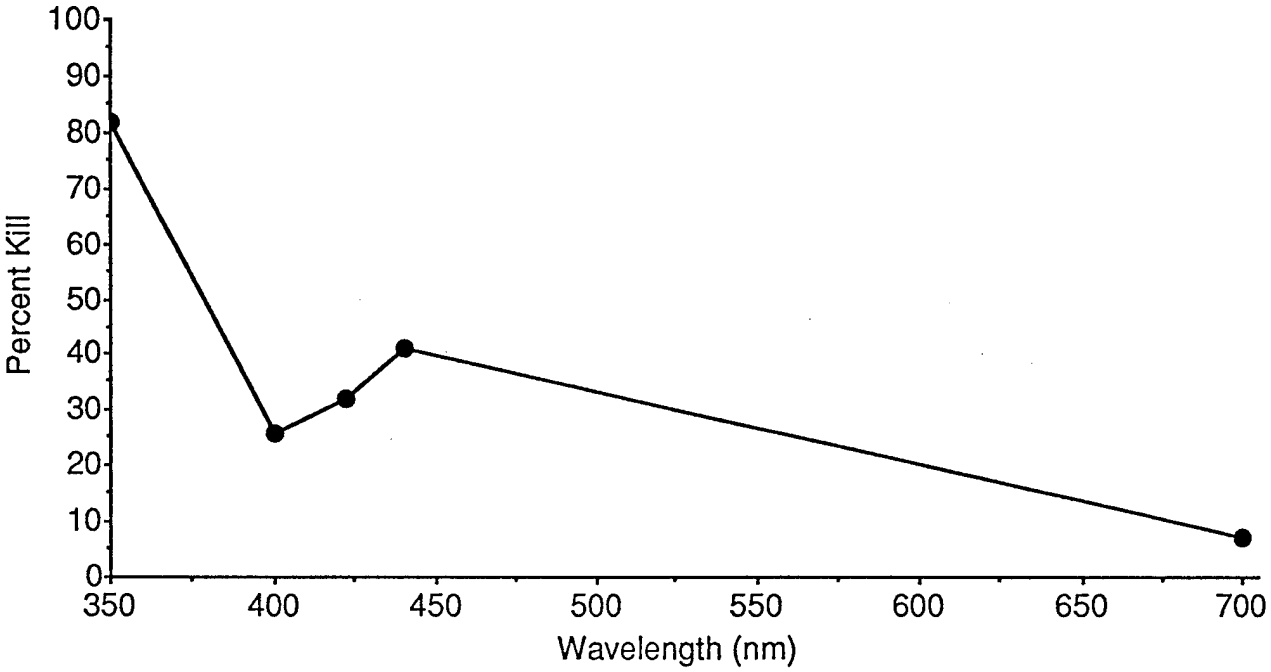

Percent Kill
100
90
80
70
60
50
40
30
20
10
0
350
400
450
500
550
600
650
700
Wavelength (nm)

Figure 5